Supplemental Figures and Legends

Supplemental Figure S1

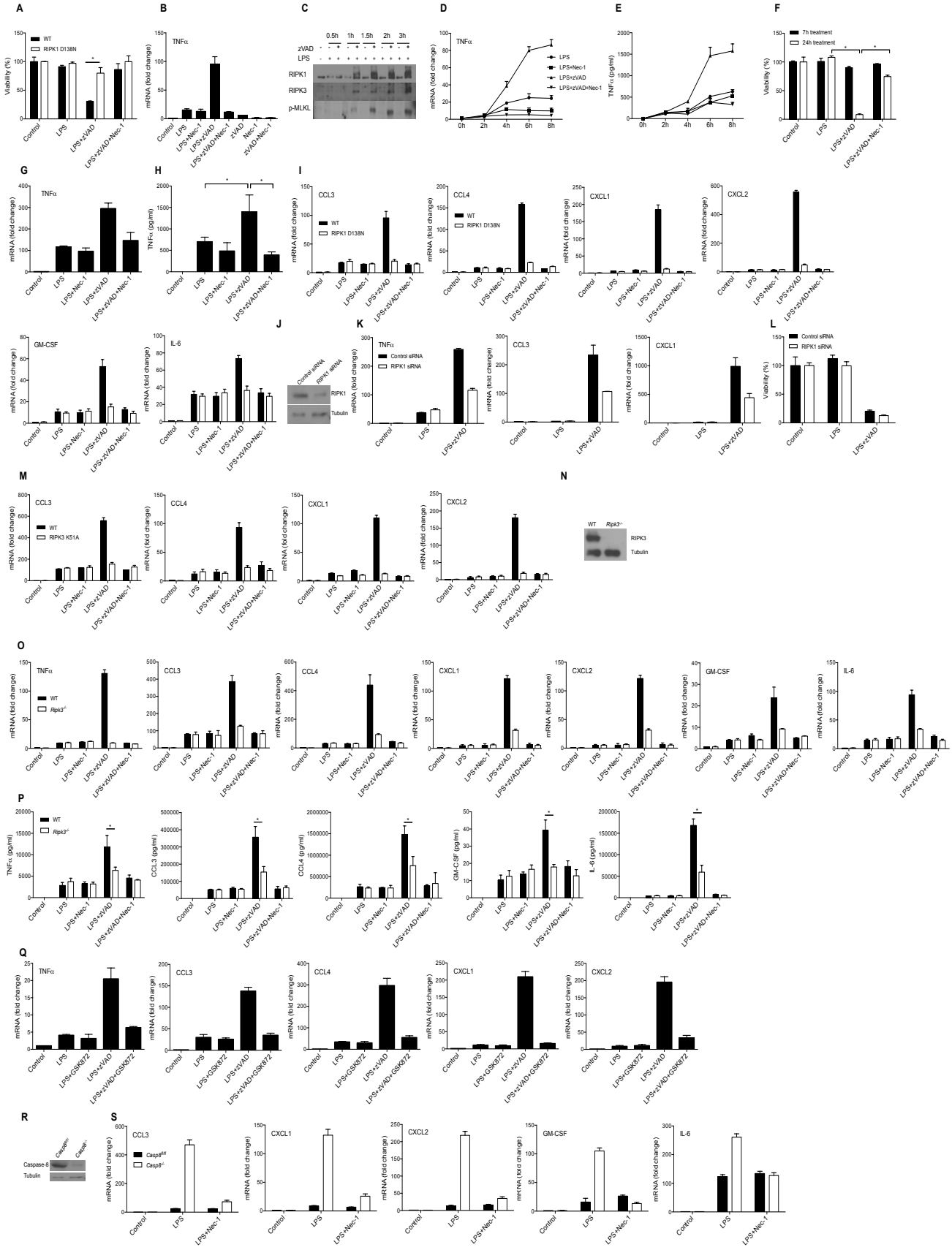

Figure S1. LPS induces RIPK1 and RIPK3 kinase-dependent cytokine production in the absence of Caspase-8 activity *in vitro*. Related to figure 1.
(A) Cell viability of wild type and D138N RIPK1 BMDMs treated for 24 hr. (B) qRT-PCR analysis of TNF mRNA expression in wild type BMDMs treated as indicated for 7 hrs. (C) Western blot analysis of NP40 insoluble fractions probed for RIPK1, RIPK3, and p-MLKL in wild type BMDMs treated for 3 hrs. (D-E) qRT-PCR and ELISA analysis of the time course of TNF mRNA expression (D) and protein release (E) in wild type BMDMs over 8 hrs. Time course was repeated twice and data are representative. (F) Cell viability of wild type BMDMs treated for 7 or 24 hrs. (G-H) qRT-PCR and ELISA analysis of TNF mRNA expression (G) and protein release (H) in wild type thioglycollate-elicited peritoneal macrophages treated for 7 hrs. (I) qRT-PCR analysis of mRNA expression of select inflammatory cytokines in wild type and D138N RIPK1 BMDMs treated for 7 hrs. (J-L) Analysis of mRNA expression of select inflammatory cytokines (K, 7 hrs) and cell viability (L, 24 hrs) in BMDMs following siRNA silencing of RIPK1. Silencing of RIPK1 was confirmed by Western blot (J). Scrambled siRNA was used as a control. (M) qRT-PCR analysis of mRNA expression of select inflammatory cytokines in wild type and K51A RIPK3 BMDMs treated for 7 hrs. (N-P) qRT-PCR and ELISA analysis of mRNA expression (O) and protein release (P) of select inflammatory cytokines in wild type and *Ripk3*$^{-/-}$ BMDMs treated for 7 hrs. Loss of RIPK3 protein confirmed by Western blot (N). (Q) qRT-PCR analysis of mRNA expression of select inflammatory cytokines in wild type BMDMs treated for 7 hrs in the presence of RIPK3 kinase inhibitor, GSK872. (R-S) qRT-PCR analysis of mRNA expression of select inflammatory genes in *Casp8*$^{flox/flox}$ BMDMs treated for 7 hrs following Adenoviral-Cre-GFP-mediated excision of *casp8*. Adeno-GFP virus was used as a negative control. Excision of C*asp8* by Adenoviral-Cre-GFP was confirmed by Western blot (R).
For qRT-PCR and Western blots, data for one representative experiment out of at least three independent repeats are shown. For ELISA, combined data for three independent experiments are shown unless otherwise stated. Values represent Mean ± SD. *$p<0.05$.
Cells were treated with LPS=10 ng/ml, zVAD=50 μM, Nec-1=30 μM and GSK872=5 μM.

Supplemental Figure S2

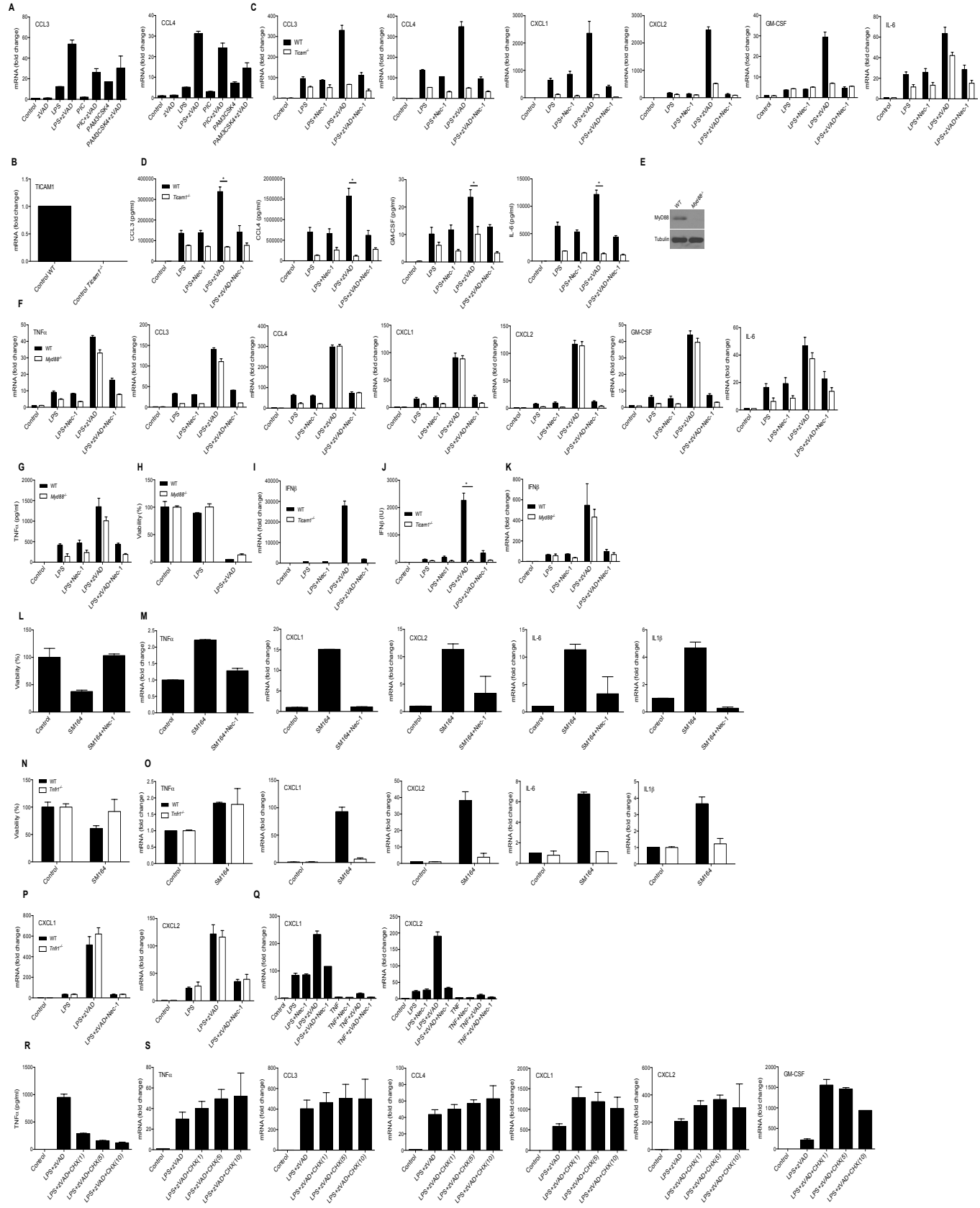

Figure S2. RIPK1 kinase-dependent cytokine synthesis induced by LPS with zVAD is dependent on TRIF but independent of MyD88 and Tnfr1. Related to figure 2.

(A) qRT-PCR of mRNA expression of CCL3 and CCL4 in wild type BMDMs treated with zVAD and/or TLR4 agonist (LPS), TLR3 agonist (Poly(I:C)), or TLR2 agonist (Pam_3CSK_4), for 7 hrs. (B-D) qRT-PCR and ELISA analysis of mRNA expression (C) and protein release (D) of select inflammatory cytokines in wild type and *Ticam1*$^{-/-}$ BMDMs treated for 7 hrs. Lack of wild type TRIF mRNA expression in *Ticam1*$^{-/-}$ BMDMs was confirmed by qRT-PCR (B) using the recommended protocol by Jackson labs. (E-G) qRT-PCR and ELISA analysis of mRNA expression of select inflammatory cytokines (F) and TNF protein release (G) in wild type and *Myd88*$^{-/-}$ BMDMs treated for 7 hrs. (E) Loss of Myd88 protein confirmed by Western blot. (H) Cell viability of wild type and *MyD88*$^{-/-}$ BMDMs treated for 24 hrs. (I) qRT-PCR analysis of IFNβ mRNA expression in wild type *Ticam1*$^{-/-}$ BMDMs. (J) ELISA analysis of IFNβ protein release in wild type *Ticam1*$^{-/-}$ BMDMs. (K) qRT-PCR analysis of IFNβ mRNA expression in wild type *MyD88*$^{-/-}$ BMDMs. (L) Cell viability of BALB/C BMDMs treated with SMAC mimetic, SM164, for 9 hours. (M) qRT-PCR analysis of mRNA expression of select inflammatory cytokines in BALB/C BMDMs treated with SM164 for 6 hrs. (N) Cell viability of wild type and *Tnfr1*$^{-/-}$ BMDMs treated with SM164 for 20 hrs. (O) qRT-PCR analysis of mRNA expression of select inflammatory cytokines in wild type and *Tnfr1*$^{-/-}$ BMDMs treated with SM164 for 6 hrs. (P) qRT-PCR of mRNA expression of CXCL1 and CXCL2 in wild type and *Tnfr1*$^{-/-}$ BMDMs treated with LPS or LPS with zVAD for 7 hrs. (Q) qRT-PCR of mRNA expression of CXCL1 and CXCL2 in wild type BMDMs treated with either LPS with zVAD or TNF with zVAD for 7 hrs. (R) ELISA analysis of TNFα protein release in wild type BMDMs treated for 4 hrs. (S) qRT-PCR analysis of select inflammatory cytokines in wild type BMDMs treated with LPS with zVAD in the presence of increasing concentrations of cycloheximide (CHX) for 4 hrs.

For qRT-PCR and Western blots, data for one representative experiment out of at least three independent repeats are shown. For ELISA, combined data for three independent experiments are shown. Values represent Mean ± SD. *$p<0.05$. Experiments using SM164 or CHX were repeated twice and data shown are representative. Cells were treated with LPS=10 ng/mL, zVAD=50 μM, Nec-1=30 μM, SM164=5 μM, CHX=1, 5, or 10 μg/mL.

Supplemental Figure S3

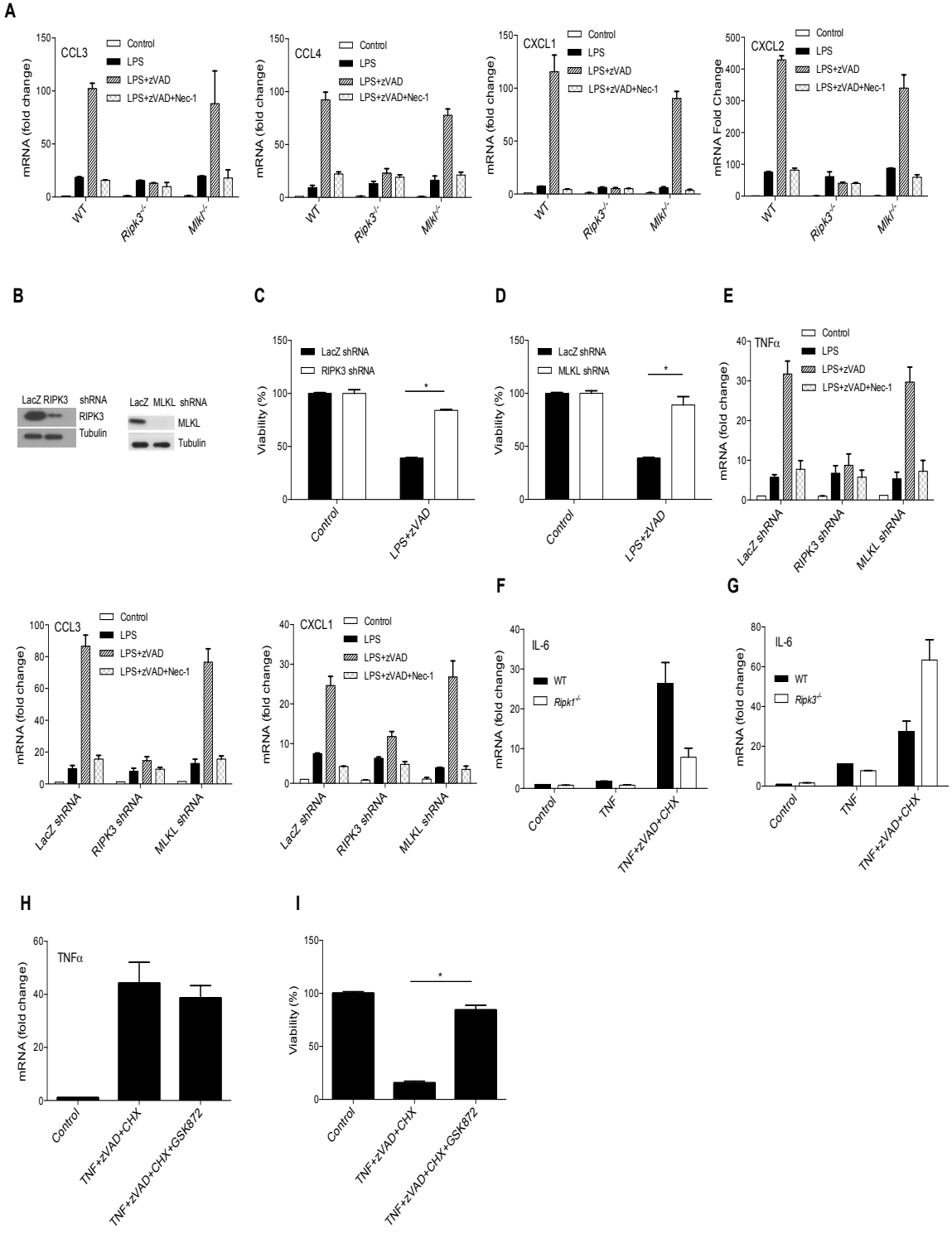

Figure S3. MLKL is dispensable for RIPK1 and RIPK3 kinase-dependent inflammatory cytokine synthesis induced by LPS with zVAD and RIPK3 regulates cytokine production in a context dependent manner. Related to figure 3.

(A) qRT-PCR analysis of mRNA expression of select inflammatory cytokines in wild type, *Mlkl*$^{-/-}$, and *Ripk3*$^{-/-}$ BMDMs treated for 7 hrs. (B) Silencing of RIPK3 and MLKL using targeted shRNA in iBMMs confirmed by Western blot. (C-D) Cell viability iBMMs expressing RIPK3 (C) and MLKL (D) shRNAs treated with LPS alone LPS with zVAD, or LPS with zVAD and Nec-1 for 24 hours. (E) qRT-PCR analysis of mRNA expression of select inflammatory cytokines in iBMMs expressing RIPK3 and MLKL shRNAs treated for 7 hours. (F-G) qRT-PCR analysis of IL6 mRNA expression in wild type and *Ripk1*$^{-/-}$ MEFs (F) and *Ripk3*$^{-/-}$ MEFs (G) treated with TNF, zVAD, and CHX for 4 hrs. (H-I) qRT-PCR analysis of TNF mRNA expression (H, 4 hrs) and cell viability (I, 24 hrs) analysis in wild type MEFs treated with TNF, zVAD, and CHX in the presence of GSK872..

For qRT-PCR and Western blots, data for one representative experiment out of at least three independent replicates are shown. For viability, combined data for three independent experiments are shown. Values represent Mean ± SD. *$p<0.05$. BMDMs and iBMMs were treated with LPS=10 ng/ml, zVAD=50 μM, Nec-1s=30 μM and/or GSK872=5 μM. MEFs were treated with TNF=10 ng/ml, zVAD=25 μM, CHX=1 μg/m and GSK872=5 μM.

Supplemental Figure S4

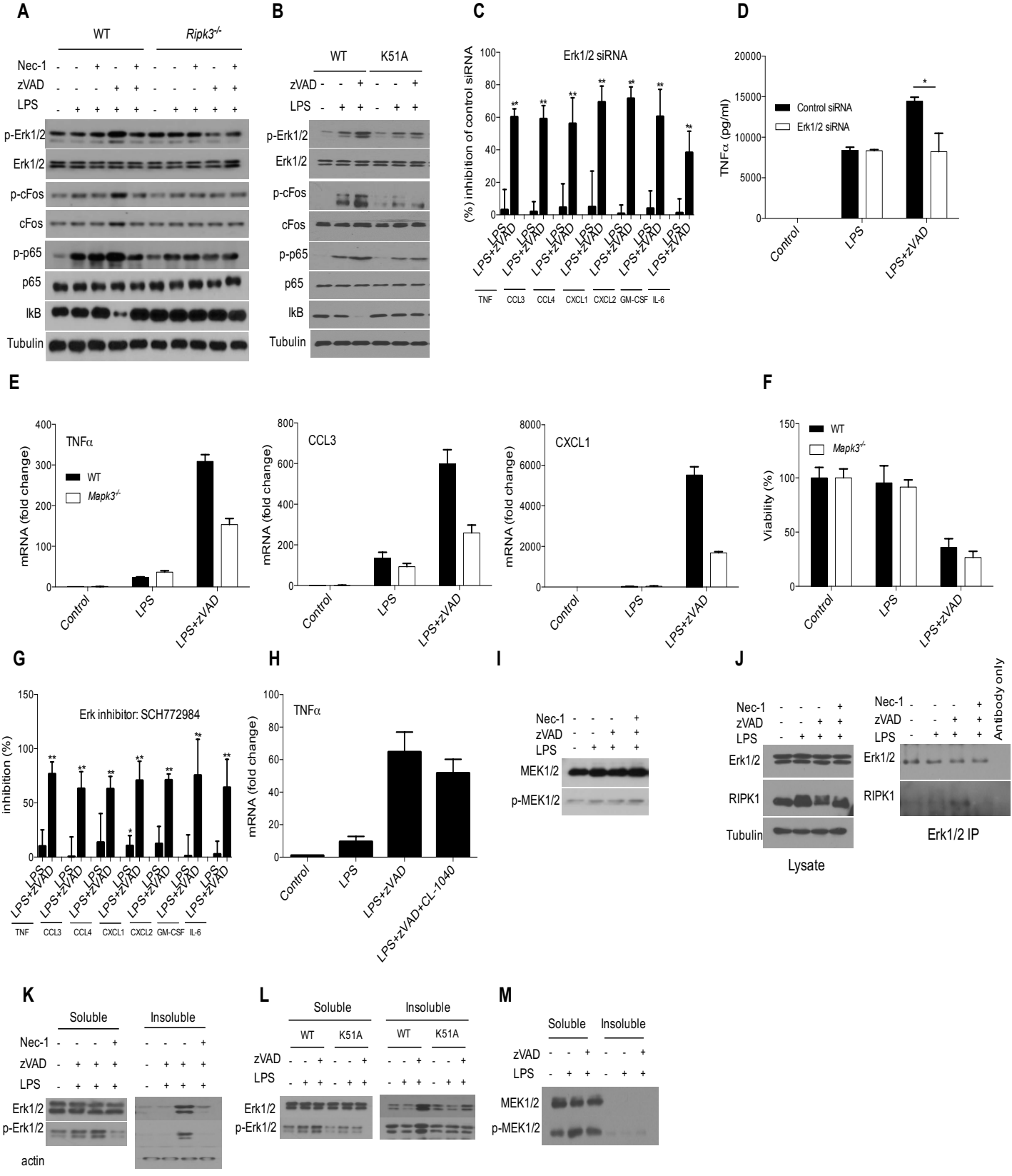

Figure S4. Inhibition of Erk1/2 blocks cytokine mRNA expression induced by LPS with zVAD. Related to figure 4.

(A-B) Western blot analysis of Erk1/2 phosphorylation in wild type, *Ripk3*$^{-/-}$ (B) and K51A RIPK3 (C) BMDMs treated for 7 hrs. (C) Percent inhibition of mRNA expression of select inflammatory cytokines in BMDMs treated with LPS or LPS with zVAD for 7 hrs following electroporation with Erk1/2 siRNA, evaluated by qRT-PCR. The data reflect the percent inhibition of cytokine mRNA expression in BMDMs electroporated with Erk1/2 siRNA compared to the cells electroporated with control siRNA. Data are the means of three biologic replicates ± SD. *$p<0.05$ and **$p<0.005$. (D) ELISA analysis of TNF protein release in BMDMs treated for 7 hrs following electroporation with Erk1/2 siRNA. (E) qRT-PCR analysis of mRNA expression of select inflammatory genes in wild type and *Mapk3*$^{-/-}$ BMDMs treated for 7 hrs. (F) Cell viability in wild type and *Mapk3*$^{-/-}$ BMDMs treated for 24 hrs. (G) Percent inhibition of mRNA expression of select inflammatory cytokines in BMDMs treated with LPS or LPS with zVAD for 7 hrs in the presence of Erk1/2 inhibitor (SCH772984), evaluated by qRT-PCR. Data reflect percent inhibition of cytokine mRNA expression in BMDMs treated with Erk1/2 inhibitor over DMSO treated controls. Data are the means of three biologic replicates ± SD. *$p<0.05$ and **$p<0.005$. (H) qRT-PCR analysis of TNF mRNA expression in BMDMs treated for 7 hrs in the presence of MEK inhibitor (CL-1040). (I) Western blot analysis of MEK1 and MEK2 as well as their phosphorylated forms in wild type BMDMs. (J) Co-immunoprecipitation of endogenous RIPK1 and Erk1/2 from BMDMs stimulated for 3 hrs. Western blot analysis for the lysate (left panel) and Erk1/2 IP (right panel) samples are shown. (K) Western blot analysis of NP40 soluble and NP40 insoluble fractions probed for Erk1/2 and phosphorylated Erk1/2 in wild type BMDMs treated with LPS alone, LPS with zVAD, or LPS with zVAD and Nec-1 for 4 hrs. (L) Western blot analysis of NP40 soluble and NP40 insoluble fractions probed for Erk1/2 and phosphorylated Erk1/2 in wild type and K51A RIPK3 BMDMs treated with LPS alone, LPS with zVAD, or LPS with zVAD and Nec-1 for 4 hrs. (M) Western blot analysis of NP40 soluble and NP40 insoluble fractions probed for MEK1/2 and phosphorylated MEK1/2 in wild type BMDMs treated with LPS alone or LPS with zVAD for 4 hrs.

For qRT-PCR and Western blots, data for one representative experiment out of at least three independent repeats are shown unless otherwise specified. For ELISA, combined data for three independent experiments are shown. Values represent Mean ± SD. BMDMs were treated with LPS=10 ng/ml, zVAD=50 μM, Nec-1=30 μM, Erk inhibitor SCH772984= 0.5 μM, and MEK inhibitor CL1040=1 μM.

Supplemental Figure S5

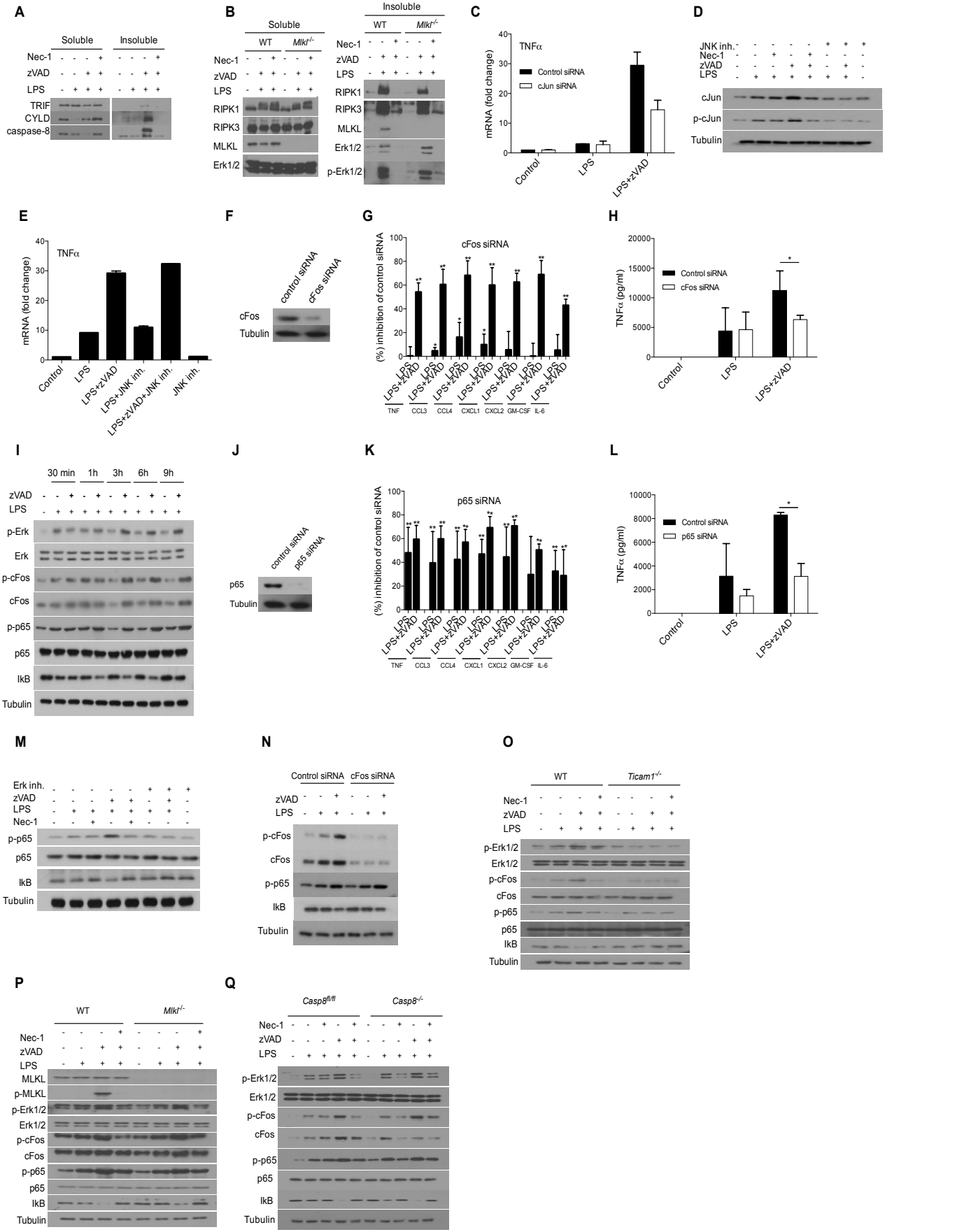

Figure S5. Inhibition of cFos and NFκB pathway blocks cytokine mRNA expression induced by LPS with zVAD. Related to figure 4.

(A) Western blot analysis of NP40 soluble and NP40 insoluble fractions probed for TRIF, CYLD, and Caspase-8 in wild type BMDMs treated with LPS alone, LPS with zVAD or LPS with zVAD and Nec-1 for 4 hrs. (B) Western blot analysis of NP40 soluble and NP40 insoluble fractions in wild type and *Mlkl*$^{-/-}$ BMDMs treated with LPS alone, LPS with zVAD, or LPS with zVAD and Nec-1 for 4 hrs. (C) qRT-PCR analysis of TNF mRNA expression in BMDMs treated for 7 hrs following electroporation with cJun siRNA. (D) Western blot analysis of cJun and phosphorylated cJun in BMDMs treated for 7 hrs in the presence of JNK inhibitor VIII. (E) qRT-PCR analysis of TNF mRNA expression in wild type BMDMs treated for 7 hrs in the presence of JNK inhibitor VIII. (F) Silencing of cFos using targeted siRNA in BMDMs confirmed by Western blot. (G) Percent inhibition of mRNA expression of select inflammatory cytokines in BMDMs treated with LPS or LPS with zVAD for 7 hrs following electroporation with cFos siRNA,evaluated by qRT-PCR. The data reflect the percent inhibition of cytokine mRNA expression in BMDMs electroporated with cFos siRNA compared to the cells electroporated with control siRNA. Data are the means of three biologic replicates ± SD. *$p<0.05$ and **$p<0.005$. (H) ELISA analysis of TNF protein release in wild type BMDMs treated with LPS with zVAD for 7 hrs following electroporation with cFos siRNA. (I) Western blot analysis of changes in Erk1/2, cFos, p65 and IkB in wild type BMDMs treated with LPS or LPS with zVAD over a 9 hr time course. (J) Silencing of p65 using targeted siRNA in BMDMs confirmed by Western blot. (K) Percent inhibition of mRNA expression of select inflammatory cytokines in BMDMs treated with LPS or LPS with zVAD for 7 hrs following electroporation with p65 siRNA, evaluated by qRT-PCR. The data reflect the percent inhibition of cytokine mRNA expression in BMDMs electroporated with p65 siRNA compared to the cells electroporated with control siRNA. Data are the means of three biologic replicates ± SD. *$p<0.05$ and **$p<0.005$. (L) ELISA analysis of TNF protein release in wild type BMDMs treated with LPS with zVAD for 7 hrs following electroporation with p65 siRNA. (M) Western blot analysis in BMDMs treated for 7 hrs in the presence of Erk inhibitor (SCH772984). (N) Western blot analysis in BMDMs treated with LPS alone or LPS with zVAD for 7 hrs following electroporation with cFos siRNA. (O) Western blot analysis in wild type and *Ticam1*$^{-/-}$ BMDMs treated with LPS alone, LPS with zVAD, or LPS with zVAD and Nec-1 for 7 hrs. (P) Western blot analysis in wild type and *Mlkl*$^{-/-}$ BMDMs treated with LPS alone, LPS with zVAD, or LPS with zVAD and Nec-1 for 7 hrs. (Q) Western blot analysis in caspase-8-floxed and caspase-8-deficient BMDMs treated with LPS alone, LPS with zVAD, or LPS with zVAD and Nec-1 for 7 hrs. *Caspase-8* was deleted from *casp8*$^{flox/flox}$ BMDMs by Adenoviral-Cre-GFP mediated excision.

For qRT-PCR and Western blots, data for one representative experiment out of at least three independent repeats are shown unless otherwise specified. For ELISA, combined data for three independent experiments are shown. Values represent Mean ± SD. BMDMs were treated with LPS=10 ng/ml, zVAD=50 μM, Nec-1=30 μM, Erk inhibitor SCH772984= 0.5 μM, and JNK inh. VIII=10 μM.

Supplemental Figure S6

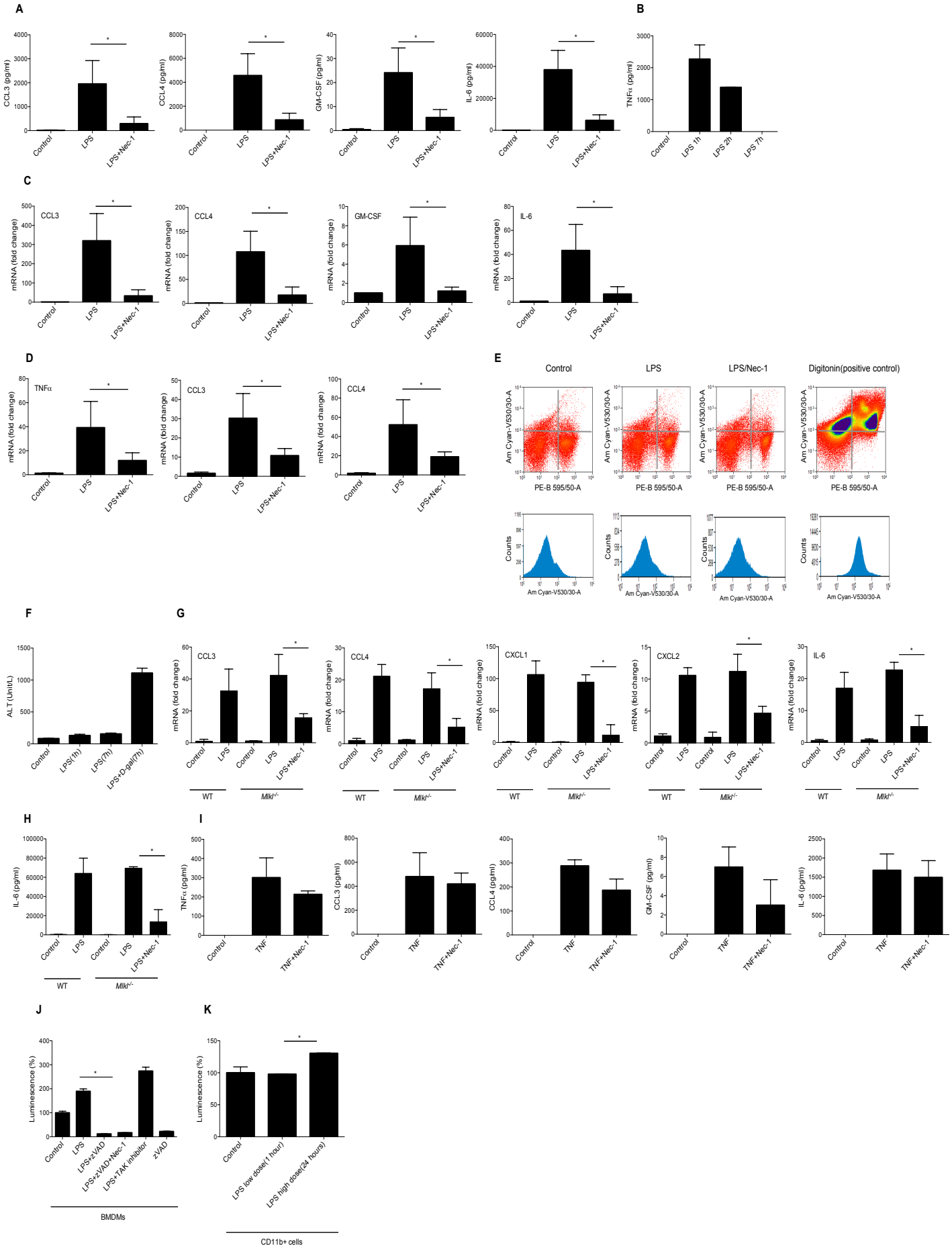

Figure S6. LPS induced inflammation requires kinase activity of RIPK1 and is independent of cell death *in vivo.* Related to figures 5 and 6

(A) Multiplexed ELISA analysis of circulating inflammatory cytokines in wild type mice injected with Nec-1s (iv) 15 min prior to LPS (ip). n= 8-9 animals per group and *p<0.05. (B) Serum TNF was measured 1, 2 and 7 hours after injection with 50 μg/kg LPS alone, n=3 per group. (C) qRT-PCR analysis of mRNA expression of inflammatory genes in $CD11b^{+}$ bone marrow cells isolated from mice post-injection with LPS or LPS/Nec-1s as in (A). (D) qRT-PCR analysis of mRNA expression of inflammatory genes in $CD11b^{+}$ thioglycollate-elicited peritoneal macrophages from mice post-injection with LPS or LPS/Nec-1s as in (A). n=3 animals per group and *p<0.05. Values represent Mean ± SD. (E) FACS analysis of $CD11b^{+}$ cells from bone marrow collected 1 hr after LPS injection. Cell viability was evaluated using LIVE/DEAD AQUA Dead Cell Stain Kit. Post-isolation treatment with digitonin was used as a positive control for cell death. (F) Serum ALT was measured 1 hr and 7 hrs after injection with 50 μg/kg LPS alone or in combination with 800 mg/kg D-gal, n=3-5 per group. (G) qRT-PCR analysis of mRNA expression of inflammatory genes in CD11b+ bone marrow cells isolated from wild type and *Mlkl*$^{-/-}$ mice injected with Nec-1 (iv) 15 min prior to LPS (ip) or LPS (ip) alone. Cells were collected 1 hr after LPS injection. n=3 animals per group and *p<0.05. (H) ELISA analysis of circulating IL6 from wild type and *Mlkl*$^{-/-}$ mice injected with LPS or LPS/Nec-1 as in (G). n=5 animals per group and *p<0.05. (I) Multiplexed ELISA analysis of select circulating inflammatory cytokines after injection of wild type mice with human TNF(50 μg/kg) or TNFand Nec-1 (30 mg/kg). Samples were collected 1 hr post-treatment, n=3 per group. (J) Caspase-8 activity assay in wild type BMDMs treated for 3 hours. n=3 biological replicates and *p<0.05. (K) Caspase-8 activity assay in CD11b+ cells, isolated and prepared as in (C and G) and (F). n=3 replicates per group and *p<0.05.

Supplemental Tables

Table S1: Similar genes are controlled by RIPK1 in BMDMs *in vitro* and in bone marrow CD11b^{+} following injection of LPS *in vivo*.
Table S1, related to figure 5.

	BMDMs *in vitro*		Bone marrow CD11+ *in vivo*	
Gene	Fold induction of LPS/zVAD to LPS alone	Fold reduction by addition of Nec-1 to LPS/zVAD	Fold induction by LPS alone	Fold reduction by addition of Nec-1
Edn1	19.49	14.77	39.68	12.10
Ifnb1	13.2	9.23	2.97	3.67
Ccl3	11.72	12.6	319.6	9.73
Csf1	9.6	5.85	2.08	1.91
Csl2	8.72	5.59	47.2	3.19
Ccl4	5.28	5.87	107.38	6.22
Cxcl1	4.74	4.65	165.98	3.35
Cxcl2	3.97	3.48	293.1	3.8
Ccl7	2.34	2.09	2.87	3.87
Il6	2.28	3.04	43.29	6.18
Csf2	2.08	2.67	5.92	4.93
Il1b	1.47	5.07	29.4	3.48
Csf	1.45	2.46	3.97	2.38

Table S1: Similar genes are controlled by RIPK1 in BMDMs *in vitro* and in bone marrow CD11b^{+} following injection of LPS *in vivo*.

Gene expression changes in BMDMs and bone marrow samples were evaluated by qRT-PCR. Upregulation by LPS with zVAD *in vitro* relative to LPS alone and

LPS with zVAD and Nec-1 is shown. Upregulation by LPS *in vivo* relative to vehicle and LPS with Nec-1 is shown.

Table S2: Major pathways induced by LPS and inhibited by Nec-1 *in vivo*. Table S2, related to figure 5.

Ingenuity pathways inhibited by Nec-1 after LPS injection	p-values
Pattern recognition of bacteria/viruses	$6.3X10^{-17}$
IL10 signaling	$1.2X10^{-13}$
NFkB signaling	$7.9X10^{-12}$
Death receptor signaling	$2X10^{-11}$
Dendritic cells maturation	$7.9X10^{-11}$
Toll-like receptor signaling	$1X10^{-10}$
IL6 signaling	$1.1X10^{-9}$
Activation of IRF3 by cytosolic DNA	$1.5X10^{-12}$

Table S2: Major pathways induced by LPS and inhibited by Nec-1s *in vivo.*

Analysis of the signaling pathways attenuated by Nec-1s in LPS-injected animals. Mice were injected with 20 mg/kg Nec-1s (iv) 15 min prior to 50 µg/kg LPS (ip). Changes in gene expression were analyzed using Ingenuity software and Fisher's exact test for p-value.

Supplemental Experimental Procedures

Animals

Female BALB/C (Charles River Labs) or C57BL/6 mice 6-8 weeks of age were used for LPS experiments. *Ripk3*$^{-/-}$ (on C57BL/6 background) and matched controls were previously described (Newton et al., 2004) and provided to us by Dr. Vishva Dixit (Genentech). *Ticam1*$^{-/-}$ (C57BL/6J-*Ticam1Lps2*/J) mice, *Myd88-/-* (B6.129-*Myd88*$^{tm1.1Defr/J}$), *Mapk3*$^{-/-}$ (B6.129-*Mapk3*$^{tm1Gela/J}$) mice and corresponding control mice (C57BL/6J, B6.129) were purchased from Jackson labs. *Tnfr1*$^{-/-}$ mice (C56BL/6-*Tnfrsf1*^{tm1Imx}*/J*) on a C57BL/6 background were obtained from Jackson labs. Floxed caspase-8 (*Casp8*$^{fl/fl}$) mice were generated on FVB/N background and were a generous gift of Dr. Stan Krajewski (Burnham institute) (Krajewska et al., 2011). RIPK1 D138N and RIPK3 K51A (*Ripk1*$^{D138N/D138N}$ *and Ripk3*$^{K51A/K51A}$) mice were previously described (Polykratis et al., 2014, Moriwaki et al., 2015). All use of animals was approved by the Tufts University, UMASS and Fox Chase Cancer Center Institutional Animal Care and Use Committees. Mice were maintained in animal facilities in cages with light/dark cycle and experiments were performed according to the protocol with all efforts to minimize the number and suffering of the animals.

Reagents

Lipopolysaccharide (LPS) (Escherichia coli 0111:B4) was purchased from Sigma and Mouse TNFα was purchased from Peprotech. Optimized Necrostatin-1 (Nec-1) (5-[(7-chloro-1H-indol-3-yl) methyl]-3-methyl-2,4-imidazolidinedione) (Nec-1s) was synthesized as previously described (Teng et al., 2005). For *in vivo*

administration, Nec-1 was dissolved in PBS containing 25% Polyethylene Glycol 400 (Spectrum labs). Endotoxin free recombinant Human TNFα was purified in Dr. Sergei Nedospasov's lab and injected at 50 µg/kg intra-peritoneal. D-(+)-Galactosamine hydrochloride (D-gal) was purchased from sigma and injected at 800 mg/kg dose intraperitoneal after dissolved in PBS. RIPK3 inhibitor, GSK872, was also previously described (Mandal et al., 2014). Other inhibitors (and their final concentrations) were as follows: Erk inhibitor, SCH772984 (0.5 μM, Selleck Chemicals), JNK inhibitor VIII (10 μM, Calbiohem), Akt inhibitor VIII (10 μM, Calbiochem), MEK1/2 inhibitors (CL1040, 1 μM, Selleck Chemicals), SM164 (5µM, Apex Bio), pan-caspase inhibitor z-Val-Ala-Asp-FMK (zVAD.fmk, 50 μM, Apex Bio).

Cells

Immortalized C57Bl/6J bone marrow derived macrophages (iBMMs) were a generous gift of Dr. Katherine Fitzgerald (UMASS Medical School). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic mixture (Invitrogen). 1 X 10^6 iBMMs were seeded for mRNA and western blot experiments into 35 mm^2 dishes and 20,000 cells per well were seeded into 96 well plates for viability. Bone marrow derived macrophages (BMDMs) were prepared from bone marrow cells collected from femurs and tibias of mice. Bone marrow cells were differentiated over 7 days in the presence of conditioned media from L929 cells (30% L929 conditioned media, 20% FBS and 1% antibiotics in RPMI1640) in petri dishes. Media was replenished on day 3. On day 7, adherent cells were collected and reseeded in tissue-culture treated

plates for experimentation. After reseeding, cells were maintained in media containing 10% L929 conditioned media, 20% FBS, and 1% antibiotics for 48 hours prior to carrying out experiments. For mRNA and Western blot experiments, $2X10^6$ BMDMs were seeded into 35 mm^2 dishes. For cell viability experiments, 50,000 cells per well were seeded in 96-well plates. Thioglycolate-elicited peritoneal macrophages were prepared by priming mice with 1 mL thioglycolate (Remel) intra-peritoneally using an insulin syringe. Mice were euthanized 3 to 4 days after injection, the abdomen was cleaned with 70% ethanol, and the peritoneal cavity was washed with 5 mL PBS using 18-gauge needle. The wash was added to a 15 ml tube containing DMEM+10%FBS and antibiotics. The contents were spun for 10 minutes at 430g, the supernatant aspirated, the pellet was resuspended in a fresh volume of DMEM+10%FBS+ 1% antibiotics and seeded. Cells were treated for experiments 24 hours post-seeding. For mRNA and Western blot experiments, 2 X 10^6 peritoneal macrophages were seeded into 35 mm^2 dishes. For cell viability experiments, 50,000 cells per well were seeded in 96 well plates. WT, $RIPK1^{-/-}$, and $RIPK3^{-/-}$ MEFs (Cho et al., 2009, Kelliher et al., 1998) were maintained in DMEM, 10% FBS and 1% antibiotics. Cells were seeded in a similar format to BMDMs for experiments.

Western Analysis

For Western analysis data, one representative experiment out of at least three independent replicates are shown. Cells were lysed and harvested in RIPA buffer (Cell Signaling) supplemented with Phenymethanesulfonylfluoride (PMSF) (5 µg/ml, Sigma), leupeptine (1µg/ml), pepstatin (1µg/ml) and aproprotinin (1µg/ml).

The protein concentrations were determined using the Pierce 660 nM Protein Assay reagent (Thermo Scientific), and equal amounts of protein were subjected to Western blotting. Samples were separated by SDS/PAGE and transferred to PVDF membranes. Membranes were blocked in Protein-free T20 (TBS) blocking buffer (Fisher Scientific) at room temperature for 1 h, and incubated at 4 °C overnight with primary antibodies (1:1000 dilution in blocking reagent). The following day, membranes were washed and incubated with secondary antibody (1:5000 dilution) at room temperature for 1 h. Signals were developed using Luminata Classico or Forte HRP substrates (Millipore).

Antibodies

The following antibodies acquired from Cell Signaling Technologies were used: NF-κB p65, p-NF- κB p65 (phospho-Ser536), IκB-α, p44/42 MAPK (Erk1/2), p-p44/42 MAPK (p-Erk1/2)(phospho-Thr202/Tyr204), cFos, p-cFos (phospho-Ser32), caspase-8 mouse specific, MEK1/2, p-MEK1/2(phospho-S217/221), αTubulin, anti-mouse IgG HRP-linked antibody, anti-rabbit IgG HRP-linked antibody and RIPK1. RIPK3 antibody was purchased from Prosci, MLKL antibody was purchased from Biorbyt, p-MLKL (pospho-Ser345) antibody was purchased from abcam. p44/42 (Erk1/2) MAP Kinase assay kit # 9800 was purchased from Cell Signaling. CYLD antibody was purchased from Thermo Fisher Scientific. TRIF antibody was purchased from Novus Biologicals. Immunoprecipitation of RIPK1 was carried out using a RIPK1 antibody purchased from BD transduction laboratories. Primary antibodies were used at a dilution of 1:1000 and secondary antibodies were used at a dilution of 1:5000.

Cell Viability Assays

Cells were seeded as described above in 100 µL of media. Typically, cells were treated with 10 ng/ml LPS and 50 µM zVAD for 24 hours. Cell viability was determined using CellTiter-Glo viability assay kit (Promega). Each independent experiment was performed in duplicate and repeated three times. Data shown is a combination of three independent replicates unless otherwise stated. Viability of the cells was analyzed relative to an untreated control.

Measurement of inflammatory molecules by ELISA

Meso Scale Discovery's (MSD) 96-Well MULTI-SPOT mouse multiplex assay was performed according to the manufacturer's instructions to determine the concentrations of TNFα, CCL3, CCL4, GMCSF and IL6 in the serum or cell culture media. Plates were read on MSD sector image 2400. Mouse TNFα was also measured using colorimetric ELISA assay (R&D Systems) according to manufacturer's protocol. Mouse interferon beta (IFN-β) was measured using colorimetric ELISA as following: 96 well plate was kept overnight in 4^0C after coating the wells with 50 µL of monoclonal rat anti-mouse IFN-β (Santa Cruz) captured antibody that was diluted 1:500 in carbonate buffer (PH 9.5, 1.6 g/L Na_2CO3 and 2.9 g/L NaHCO3). The following day, 200 µL of blocking agent with the diluent buffer (10% FBS in PBS) was added for two hours at 37^0C. Fifty µL of standard and samples were added to the wells and incubated overnight at 4^0C. Fifty µL of polyclonal rabbit anti-mouse IFN-β detecting antibody (R&D Systems) diluted 1:2000 in 10% FBS diluted in PBS was added to the wells and kept again overnight at 4^0C. Fifty µL of goat anti-rabbit-HRP secondary antibody (Cell

Signaling) diluted 1:2000 was added to the wells. Plates were incubated for 2-3 hours at room temperature. Subsequently, 50 µL of the TMB substrate was added. The reaction was stopped by the addition of 50 µL 2N H_2SO_4. Absorbance was measured at 450 nm. Blank values were subtracted. A washing step with buffer (PBS 0.05% TWEEN) was performed after each step and before the addition the substrate and stopping the reaction.

Phospho-RIPK1 ELISA

Lysates were prepared from 3 X 10^6 BMDMs and phospho-RIPK1 ELISAs were performed using multi-array 96 well plates from (Meso Scale Discovery) per the manufacturers guidelines: Capture antibody (anti-RIPK1 mAB, abcam#ab72139) was diluted to 0.5ug/ml in PBS and 35 µL/well were incubated overnight. Plates were blocked in 100µL/well blocker buffer (5% BSA diluted in PBS) while shaking for 2 hours at room temperature (RT). Twenty-five µL of each lysate sample or standard samples (RIPK1 protein, abcam, ab#135220) were added and incubated for 1.5 hour while shaking at RT. Standards were serially diluted 1:2 from 500 pg/µL to 0.122 pg/µg along with a blank. Detection antibody diluted in antibody dilution buffer (1% blocker BSA and 0.1%NP-40) was added at 1µg/mL concentration (either cell signaling RIPK1 or GSK RIPK1 p-Ser166) and incubated for 1.5 hour with shaking at RT. Secondary detection antibody (goat anti-rabbit antibody, sulfo-TAG labeled, Meso Scale Discovery, R32AB-1) diluted in antibody dilution buffer at 1µg/ml was added for 1 hour at RT with shaking Assay was developed using 150µL of MSD read buffer (R92TC-2) and plates were read on an MSD sector imager 2400.

Stable infection of iBMM cells with shRNAs

To generate retroviral shRNA constructs, shRNAs targeting mouse RIPK3 and MLKL, previously reported by Sun et al. were cloned into pLV-H1-EF1a (Biosettia) according to manufacturer's recommendations. As a control, vector encoding a shRNA against bacterial LacZ was used (Biosettia). Lentiviruses were generated by co-transfecting LentiX 293 cells in 35 mm^2 dishes with 1.28 μg of LP1, 0.64 μg LP2 and 0.8 μg LP3 constructs (Invitrogen) with 1.28 μg pLV vectors using lipofectamine 2000 transfection reagent (Invitrogen). Media (supernatants) were collected three times each 48 hr, filtered through a 0.45 um filter (Millipore) and used to infect macrophages in the presence of 8 μg/ml polybrene. Cells were selected in 1 μg/ml puromycin to create the stable shRNA cell lines.

shRNA sequences:

RIPK1 shRNA: 5'-AAAAGCATTGTCCTTTGGGCAATTTGGATCCAAATTGCCCAAAGGACAATGC-3'.

RIPK3 shRNA 5'-AAAAGCTCTCGTCTTCAACAACTTTGGATCCAAAGTTGTTGAAGACGAGAGC-3'.

LacZ shRNA: 5'-AAAAGCAGTTATCTGGAAGATCATTGGATCCAATGATCTTCCAGATAACTGC-3'

MLKL shRNA: 5'-AAAAGCTGCTTCAGGTTTATCATTTGGATCCAAATGATAAACCTGAAGCAGC-3'.

siRNA silencing

RIPK1, cFos and cJun ON-TARGET PLUS siRNA SMARTpools were purchased from Dharmacon. Erk1, Erk2 and p65 siRNAs was purchased from Cell Signaling. siRNAs were electroporated using Amaxa mouse macrophage nucleofector kit and Nucleofector II electroporator (Lonza) according to manufacturer's recommendation for primary macrophages. Macrophages were electroporated on day 4 or 5 of culture. After 72 hours, BMDMs were treated with LPS and/or LPS with zVAD for 7 hours. One hundred nM siRNAs were used for RIPK1, cJun, cFos and Erk1/2 (each) and 200 nM for p65, based on the efficiency of silencing evaluated by Western blot.

RNA extraction, cDNA synthesis and qRT-PCR

For RNA extraction, cells were seeded as described above. Cells were stimulated with 10 ng/ml LPS, 50 μM zVAD and 30 μM Nec-1 or other inhibitors as indicated. Total RNA was isolated using RNA MiniPrep kit (ZYMO Research) according to the manufacturer's protocol. 50 ng to 1 μg of RNA was converted to cDNA using iScript cDNA Synthesis Kit (Bio Rad) or ProtoScript M-MuLV First Strand cDNA Synthesis kit (New England Biolabs). . qRT-PCR reactions were performed in LightCycler 480 II using using VeriQuest SYBR Green master mix (Affymetrix) and the following program: 50°C for 2 min, 95°C for 10 min, followed

by 40 cycles of amplification (95°C for 15 sec, 60°C for 1 min). GAPDH was analyzed as a housekeeping gene.

The primer sequences used to amplify murine genes are as following:

Mouse GAPDH: forward 5'-TGTGTCCGTCGTGGATCTGA-3, reverse: 5'-GGTCCTCAGTGTAGCCCAAG3'.

Mouse TNF: forward 5'-CCCTCACACTCAGATCATCTTCT-3', reverse 5'-GCTACGACGTGGGCTACAG-3'.

Mouse CCL3: forward 5'-TTCTCTGTACCATGACACTCTGC-3', reverse 5'-CGTGGAATCTTCCGGCTGTAG-3'.

Mouse CCL4: forward 5'-TTCCTGCTGTTTCTCTTACACCT-3', reverse 5'-CTGTCTGCCTCTTTTGGTCAG-3'.

Mouse CXCL1: forward 5'-CTGGGATTCACCTCAAGAACATC-3', reverse 5'-CAGGGTCAAGGCAAGCCTC-3'.

Mouse CXCL2: forward 5'-CCACCACCAGGCTAGAGG-3', reverse 5'-GCGTCACACTCAAGCTCTG-3'.

Mouse GMCSF (CSF-2): forward 5'-GGCCTTGGAAGCATGTAGAGG-3', reverse 5'-GGAGAACTCGTTAGAGACGACTT-3'.

Mouse IL6: forward 5'-TAGTCCTTCCTACCCCAATTTCC-3', reverse 5'-TTGGTCCTTAGCCACTCCTTC-3'.

TRIF: forward 5'-GGACCTCAGCCTCTCATTATTC-3', reverse 5'-GGTTCTCCGAACACTCAGTC-3'.

Mouse CSF1: forward 5'-GGCTTGGCTTGGGATGATTCT-3', reverse 5'-GAGGGTCTGGCAGGTACTC-3'.

Mouse CSF3: forward 5'-ATGGCTCAACTTTCTGCCCAG-3', reverse 5'-CTGACAGTGACCAGGGGAAC-3'.

Mouse IFNb1: forward 5'-CAGCTCCAAGAAAGGACGAAC-3', reverse 5'-GGCAGTGTAACTCTTCTGCAT-3'.

Mouse CCL7: forward 5'-GCTGCTTTAAGCATCCAAGTG-3', reverse 5'-CCAGGGACACCGACTACTG-3'.

Microarray analysis

For Affymetrix gene chip analysis, BMDMs were treated with 10 ng/ml LPS, 50 μM zVAD and 30 μM Nec-1 for 6 hr. Total RNA was isolated using Qiagen RNeasy kit. Samples were submitted to Keck DNA microarray facility (Yale University) for hybridization and analysis using GeneChip Mouse Gene 1.0 ST Array. Functional annotation clustering of the genes, increased ≥ 1.5-fold in LPS with zVAD vs. LPS treated cells, was performed using NIH DAVID. Hierarchical clustering of the identified cytokine and/or chemokine cluster was performed using Cluster3-TreeView within R platform. Red – upregulation.

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE72797

Co-immunopricipitaion

Co-IP of endogenous RIPK1 and Erk1/2 was performed using $1x10^7$ BMDM cells per condition in 10 cm^2 dishes. Cells were lysed in a buffer composed of 30mM TrisHCl (pH7.4), 150 mM NaCl, 10%(v/v) glycerol, 10%(v/v) Triton X-100, 5 mM NaF, 10 mM sodium pyrophosphate, 175mM β-glycerol phosphate, 50μg/ml PMSF,1μg/ml leupeptine, 1μg/ml pepstatin and 1μg/ml aprotinin. Lysates were incubated overnight with 1.25 μg mouse monoclonal anti-RIPK1 antibody (BD

Biosciences) or Erk1/2 antibody (cell signaling) at 4°C. Following, lysates were incubated with 20 μL of protein G Dynabeads per reaction (Invitrogen) for 3-4 hours. Bead-protein-antibody mixtures were washed 3 times using lysis buffer with higher NaCl (500 mM). Bound proteins were eluted using SDS then heating up the samples for 5 min. Beads were retrieved using magnetic stand and solubilized protein were analyzed by Western blotting.

Necrosome formation assay

Isolation of NP40 soluble and insoluble fractions was performed as previously described (Li et al., 2012, Moquin et al., 2013). Cells were seeded into 35 cm^2 dishes at 2 X 10^6 cells/well and stimulated with 10 ng/ml LPS, 50 μM zVAD and 30 μM Nec-1 for up to 3 hr. Cells were lysed in 1%NP-40 lysis buffer (150mM NaCl, 20mM Tris-Cl (pH 7.5), 1% NP-40, 1mM EDTA, 3mM Na-fluoride, 1mM B-glycerophosphate, 1mM Sodium Orthovanadate, 5uM Idoacetamide, 2uM N-ethylmaleimide and 5μg/ml PMSF and 1μg/ml leupeptine, 1μg/ml pepstatin and 1μg/ml aprotinin. Lysates were flash frozen on dry-ice, thawed on ice and vortexed for 10 sec followed by centrifugation at 1000g for 10 minutes to remove nuclear pellets. Supernatants were collected and centrifuged at 34400g for 15 minutes in the refrigerated table-top centrifuge. Resultant supernatants were collected (NP-40 soluble fractions) and pellets (NP-40 insoluble) were boiled in 1x SDS-PAGE buffer.

Generation of *Casp8*$^{-/-}$ BMDMs using GFP-CRE adenovirus

Recombinant adenovirus expressing Cre recombinase was purchased from SignaGen Laboratories. Casp8$^{fl/fl}$ BMDMs were infected at 100-1000 MOI on Day

3. Amount of adenovirus required for complete deletion of caspase-8 (*Casp8*$^{-/-}$) (Western blot) and 100% infection efficiency (GFP fluorescence) was determined in preliminary titration experiments. Media containing virus was then removed and fresh culture media was added. Twenty-four hours later, the second infection (day 5) was done. Culture media was changed again after 24 hours and cells were treated on day 7 of BMDM differentiations. A recombinant control adenovirus expressing just GFP was used as a control. Detection of infected cells was monitored by manual visualization of GFP-positive cells using fluorescence microscopy.

***In vivo* LPS challenge and Nec-1 treatment**

Mice were injected intravenously via tail vein with 100μL of 30 mg/kg optimized Nec-1 (7-Cl-O-Nec-1), (Degterev et al., 2013) 15 min prior to intraperitoneal injection of 50 μg/kg LPS (Sigma) dissolved in 500μL PBS. Nec-1 was dissolved in PBS containing 25% Polyethylene Glycol 400 by water sonication for 15 min. Blood was collected 1 hour after LPS injection and cytokines were measured using multiplexed ELISA assay (Meso Scale Discovery) or colorimetric ELISA assay. Bone marrow cells were flushed from femurs and tibias with PBS, centrifuged at 430g, resuspended/blocked with 1 μM EDTA and 2% BSA in PBS for 45 min at 4^0C, and stained using anti-CD11b$^+$-PE antibody (Biolegend, clone M1/70, 1:500) for 60 min at 4^0C. CD11b+ cells were sorted by fluorescence-activated cell sorting (FACS) using MoFlo sorter (Beckman-Coulter).

Measurement of cell death of CD11b+ cells

LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Invitrogen, L34957) was used. $CD11b^+$ cells were collected as described previously (see: LPS Challenge), and then aqua stain was added according to manufacturer's guidelines. Cell death analysis was carried out by flow cytometry to quantify dye uptake by $CD11b^+$ cells.

Measurement of plasma alanine aminotransferase

Serum was obtained from whole blood samples centrifuged at 16500 g for 10 minutes at room temperature. Serum alanine aminotransferase (ALT) was measured to assess hepatic tissue damage using the pointe scientific, INC kit according to the recommended protocol.

Caspase-Glo Assay system

Cells were seeded in 384 well plate in 40µL of media; BMDMs were seeded at 20,000 cells per well for BMDMs and $CD11b^+$ cells were seeded at 200,000 cells per well. Luminescent assay was determined using 15 uL of Caspase-Glo reagent (Promega) after 45 min. For BMDMs, each independent experiment was performed in triplicate and repeated three times. $CD11b^+$ Caspase-Glo luminescent assay was performed with three replicates. Luminescence was analyzed relative to untreated controls.

Next Generation Sequencing

For RNA-Seq analysis, mice were divided into 3 groups - control (n=2), LPS (n=2) and LPS/Nec-1 (n=2). Bone marrow cells were isolated by FACS as described for qPCR analysis. Total RNAs were isolated using Qiagen RNeasy kit according to the manufacturer's protocol. Input RNA samples were analyzed by an Agilent

BioAnalyzer 2100 to asses the integrity and quantity of sample. RNA samples were then amplified using NuGen Ovation RNA System V2. The resulting cDNA samples were fragmented on Covaris M220 Focused Solicitor, followed by purification and concentration with a Qiagen MiniElulte Spin Column. Following this step, S1 Nuclease (Promega) was used according to the manufacture's protocol. Resulting amplified and fragmented cDNA samples from RNA amplification were used as input for library preparation, using Illumina TruSeq DNA Sample Preparation Kit per the manufacturer's instruction. The resultant libraries were quantified and pooled at equal molar concentration for sequencing. Sequencing was done on a lane of High Output single read 100 bases on an Illumina HiSeq 2500 using SBS V3 chemistry. The base calling and demultiplexing was performed with CASAVA v1.8. Resulting data were aligned to mouse mm10 reference genome with Tophat 2 and different gene expression analysis with Cuffdiff.

Gene expression profiles were analyzed using Ingenuity pathway analysis (IPA) software to identify pathways regulated by LPS and Nec-1.

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73836

Statistics

Statistics were reported for all experiments with 3 or more biological replicates, and analyzed by two-tailed Student t-test or one-way ANOVA, where specified. Statistical significance was determined using an alpha value of 0.05. All experiments were repeated with three independent biological replicas. For qRT-

PCR data, one representative data set is typically shown due to the variability in responses to LPS and/or LPS with zVAD in independent BMDM preparations**.**

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